Charge-cluster-to-alanine scanning of UL128 for fine tuning of the endothelial cell tropism of human cytomegalovirus

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Abstract:
The viral genes UL128, UL130 and UL131A have been identified as major determinants of endothelial cell (EC) tropism of human cytomegalovirus (HCMV), deletion of either gene causing a null phenotype. We hypothesized that a functional scanning of these genes by minor genetic modifications would allow for the generation of mutants with an intermediate phenotype. By combining charge-cluster-to-alanine (CCTA) mutagenesis with markerless mutagenesis of a BAC-cloned endotheliotropic HCMV-strain, we analyzed UL128 in order to identify functional sites and hence enable targeted modulation of the EC tropism of HCMV. A total of 9 mutations in 8 charge clusters were tested. Three of the CCTA mutations severely reduced EC tropism, three were irrelevant, two had a weak effect on cell tropism, and one mutation in the most C-terminal cluster caused an intermediate phenotype. All of the highly effective mutations were located in a core region (amino acids 72 to 106) which appears to be particularly crucial for EC tropism. The intermediate effect of mutations in the C-terminal cluster could be modulated by varying the number of amino acids replaced with alanine. This study provides a rational approach for targeted modulation of HCMV cell tropism, which may aid in the development of HCMV strains with a desired degree of attenuation.
Introduction

Human cytomegalovirus (HCMV) is a herpesvirus causing lifelong persistent infection which is usually clinically silent in the immunocompetent host. Under conditions of immunosuppression however, reactivation of endogenous virus or exogenous reinfection may cause viral replication and dissemination with clinical disease manifestations predominantly in the lung, the retina and the gastrointestinal tract (17). In addition, HCMV may replicate in various organs including brain, salivary glands, liver, pancreas, kidney and genital tract on a subclinical level. This systemic distribution is facilitated by a very broad cell tropism including epithelial cells, fibroblasts, smooth muscle cells, endothelial cells (ECs) and hematopoietic cells. Granulocytes, monocytes, dendritic cells (DCs) and vascular ECs are presumed to contribute particularly to hematogenous dissemination (24). Tropism for these cell types is therefore considered a pathogenicity factor of HCMV (8, 10, 24).

During long term adaptation to fibroblast cultures the tropism for ECs, granulocytes and monocytes/macrophages and DCs is lost, whereas propagation in EC cultures maintains the broad cell tropism of clinical isolates (20, 25, 28, 30). The genetic mechanism underlying fibroblast-adaptation is a modulation within the viral genes UL128, UL130 and UL131A which may affect any of these genes (7). These three genes were shown to be essential for infection of ECs, granulocytes, epithelial cells and DCs by HCMV (9, 11, 31). Recently, the UL128-131A-encoded proteins were shown to be binding partners for the glycoproteins gH and gL which were previously described to be complexed with gO in the glycoprotein complex gCIII present in the viral envelope (22, 32). Whereas fibroblast-adapted strains only bear the gH/gL/gO complex, highly endotheliotropic strains additionally contain a gH/gL/pUL128-131A complex in their envelope (13, 16,
The latter complex probably has a function for entry of HCMV into ECs, which is dispensable for entry into fibroblasts (21, 23).

Disruption of any of the UL128-131A genes results in an almost complete loss of EC tropism (1, 7, 11, 26). A more detailed analysis of the individual genes, e.g. definition of functional sites within the respective proteins, is not available, which may be due to the fact that highly efficient methods for markerless introduction of minor mutations into BAC-cloned HCMV genomes have only recently been developed (29, 33). Along with these technical constraints, mutants with intermediate phenotypes were not available. Such intermediate variants appear particularly desirable in the context of vaccine development. Previous vaccine trials with the low-endotheliotropic HCMV strain Towne had limited success (2) possibly because it was restricted to local replication (19). On the other hand, strains which have retained a high dissemination capacity are assumed to be more virulent (19). Strains with a moderate dissemination capacity may be the solution to this problem, by combining reduced virulence with sufficient potential of replication and spread.

HCMV strain TB40/E which combines high EC tropism with high productivity has recently been cloned as a bacterial artificial chromosome (BAC) in order to facilitate genetic modifications, and the particular role of the viral UL128 gene for cell tropism of this strain has been demonstrated by genetic marker transfer (26).

Based on biochemical analyses, Ryckman et al. have recently suggested a model for the protein-protein-interactions within the gH/gL/UL128-UL131A complex. In this model, gH binds both gL and pUL130 which in turn hold pUL128 in an exposed position (22). This fits well with functional data from Patrone et al. showing binding of pUL128 to the surface of ECs and inhibition of EC infection by soluble pUL128 (18), thus emphasizing
the particular role of pUL128 within the complex. Now we aimed to identify sites within pUL128 which are relevant for EC tropism by combining the charge-cluster-to-alanine (CCTA) scanning approach (12) with a markerless mutagenesis technique (29). CCTA scanning is based on the assumption that clusters of charged amino acids are likely to be exposed sites within the tertiary structure of a protein while nonpolar amino acids tend to cluster on the inside of the protein (4, 5). Charge clusters therefore have a high probability of constituting functional interaction sites of a protein. We hypothesized that by identification and targeted modification of such functional sites within UL128 it is possible to generate mutant viruses with a desired degree of EC tropism.

Materials and Methods

Cells and viruses

Human foreskin fibroblasts (HFFs) were cultured in MEM (GIBCO/Invitrogen) containing 5 % FCS, 2.4 mmol/l glutamine, 100 µg/ml gentamicin and 0.5 ng/ml bFGF. Human umbilical vein endothelial cells (HUVECs) were isolated from umbilical veins by chymotrypsin treatment. For experiments HUVECs from five different sources were pooled in order to minimize source-dependent variations. HUVECs were cultured in RPMI1640 (GIBCO/Invitrogen) with 50 µg/ml ECGS (Becton Dickinson), 10 % human serum (HCMV seronegative), 5 IU/ml heparin and 100 µg/ml gentamicin. HCMV strain TB40-BAC4 and derived mutants were propagated in HFFs. For preparation of virus stocks infectious supernatants from HFF cultures were harvested at days 5-7 p. i. Cellular debris was removed by centrifugation at 3220 x g for 10 min and the supernatants were stored at -80 °C.
Markerless mutagenesis of HCMV genomes

The highly endotheliotropic HCMV-TB40/E-derived BAC TB40-BAC4 (26) was used for the generation of mutant strains. The mutant BACs were generated with the markerless mutagenesis protocol as described by Tischer et al (29). Recombination fragments were generated by PCR from plasmid pEP-Kan-S (for primer sequences see supplementary data). The resulting recombination fragments consisted of the 18 bp I-Sce I restriction site and a kanamycin resistance cassette flanked on both sides by overlapping HCMV homologies containing the sequence of interest. For charge-cluster-to-alanine (CCTA) mutations the codons for charged amino acids in the sequence of interest were exchanged against codons for alanine. The respective recombination fragments for the different mutants were inserted into TB40-BAC4 by homologous recombination in E. coli strains SW102 (33) or GS1783 (kindly provided by Gregory A. Smith, Northwestern University, Chicago, IL). After successful kanamycin selection all non-HCMV sequences were removed by an intrabacterial I-Sce I digest and a subsequent Red recombination. Correct mutagenesis was confirmed by sequencing. The same method was applied for the generation of revertant viruses with restored wild type sequence. For virus reconstitution BAC-DNA was isolated using the NucleoBond Xtra Midi kit (Macherey-Nagel) and transfected into HFFs using the MBS transfection kit (Stratagene). Cells were propagated until viral plaques appeared.

Measurement of the endothelial cell tropism

Infectious supernatant from fibroblast cultures was tested for cell-free infectivity in HUVECs and HFFs. 2x10⁴/well HFFs and HUVECs were seeded in 96well plates coated with 0.1 % gelatin one day prior the experiment. The cells were pre-incubated for 30
minutes at 37 °C with MEM and then infected with the respective virus suspension for 1 hour. The medium was changed and the cultures were incubated over night at 37 °C. Cells were then fixed with 80 % acetone and stained for viral immediate early antigen (pUL122/123) by subsequent incubation with antibody E13 (Biosoft) and Cy3-conjugated goat anti-mouse IgG F(ab)2 (Jackson ImmunoResearch). The nuclei were counterstained with DAPI. The infection efficiency in HFFs and HUVECs was quantified and the relative EC tropism of the mutant viruses in comparison to the wild type HCMV-TB40-BAC4 was determined by relating the infection efficiency in HUVECs to the infection efficiency in HFFs.

Cell-associated viral spread in EC monolayers was tested by a focus expansion assay essentially as described previously (27). Infected fibroblasts were cocultured with uninfected ECs for 5 days in gelatin-coated 96 well plates, then fixed and stained for immediate early antigen and DAPI. The average number of infected cells per focus (FEHUVEC) was quantified.

For mutants BAC4-UL128ccta18-21, 38-42, 72-74, 155-157 and 163-165 two independent clones and for mutant BAC4-UL128ccta154-158 three independent clones were phenotypically tested. The clones of a respective mutation site resembled each other with regard to EC tropism. The shown data represents average values of all clones from at least three independent experiments. For mutations UL128ccta60-62, 82-86, 104-108 and 136-139 only single clones were available. To exclude that second site mutations within UL130 and UL131A might contribute to the observed phenotype of these clones, we sequenced these genes in addition to UL128. No single mutation - except the desired CCTA mutations in UL128 - was found in any of the tested clones.
Statistical analysis:
Statistical significance of differences in cell tropism values of the various viruses was determined using two-tailed Mann-Whitney U test analyses. When the p-value was < 0.05 the difference was considered significant, when the p-value was < 0.001 the difference was considered highly significant.

Results
Identification of charge clusters within UL128 and generation of mutants thereof
In order to modify the EC tropism of HCMV-TB40-BAC4, clusters of charged amino acids within the UL128 open reading frame were replaced individually with alanines. Exchange of charged amino acids against alanine will most probably preserve the overall protein structure but specifically destroy putative interaction sites on the surface of the protein. Therefore, CCTA scanning allows for the identification of specific protein-protein interaction sites while avoiding (though not excluding) unspecific effects on protein function due to destruction of the protein backbone (12). A charge cluster was defined as two or more charged amino acids in a window of five amino acids (34). According to this definition, eight charge clusters were identified in pUL128 (Fig. 1). In principle, the markerless mutagenesis technique as described by Tischer et al. allows for short insertions of up to 30 bp (29). For our experimental approach however, we chose to limit the extent of mutations to 15 bp to ensure efficiency. Hence, a sequence of 3-5 amino acids preferentially from the beginning or end of each charge cluster was chosen for CCTA mutagenesis, including amino acids (aa) DHSHR\textsubscript{18-21}, HPPER\textsubscript{38-42}, DGE\textsubscript{60-62}, EIR\textsubscript{72-74}, HSLTR\textsubscript{82-86}, EADGR\textsubscript{104-108}, EYDK\textsubscript{136-139}, and RAK\textsubscript{163-165}. In addition, the pentapeptide KKHKR\textsubscript{154-158} located in the middle of the last charge cluster was chosen.
for CCTA mutagenesis because of the striking accumulation of five basic amino acids in a row (Table 1). The generation of mutants was performed in *E. coli* by markerless replacement of the selected charged amino acids with alanines in BACs containing the genome of the highly-endotheliotropic HCMV-TB40-BAC4 (26). For primer sequences see Table 2. The gross integrity of the BACs was checked by restriction fragment analysis to exclude major rearrangements within the genomes (Fig. 2). For reconstitution of virus, primary human fibroblasts (HFFs) were transfected with the mutant BAC-DNAs and then monitored for appearance of cytopathic effects (CPEs). As expected from the fact that UL128 is only necessary for replication in ECs, all of the mutants yielded viral plaques in HFFs within 5-10 days after transfection, grew to 100 % CPE within two further cell culture passages, and finally produced virus titers of > 10^6 infectious units per ml, thus perfectly resembling the reconstitution of wild type HCMV-TB40-BAC4. Correctness of the recombinations was controlled in every mutant by sequencing UL128 both of the mutant BACs and of the reconstituted viruses. Stocks of each virus were then produced in HFFs and stored at -80 °C for phenotypic testing in ECs.

Endothelial cell tropism of HCMV-TB40-BAC4-UL128ccta mutants

To evaluate the effect of the various mutations on replication of the respective viruses in ECs, two independent assays were performed: (i) Cell-free virus preparations were tested on HUVECs and HFFs in parallel and the infection rate in both cell types was compared, thus revealing the "relative EC tropism" of each mutant. (ii) Infected HFFs were cocultured with an excess of HUVECs for 5 days allowing for focal spread of the virus, and the number of infected cells per focus was counted, thus yielding the "focus
expansion capacity” (FEHUVEC) as an absolute value for the ability of a mutant to replicate and spread in ECs.

For quantification of the relative EC tropism of cell-free virus preparations, virus input was normalized in HFFs for all mutants and the infection rate in ECs was then determined with the same amount of input virus (Fig. 3A and B). The relative EC tropism (infection rate in HUVECs/ infection rate in HFFs) of wild type virus was 70 % and this value was not outnumbered by any of the mutants. CCTA mutations of aa DHSR18-21, HPPER38-42 and EYDK136-139 had no effect on EC tropism, CCTA mutations of aa EIR72-74, HSLTR82-86 and EADGR104-108 resulted in a severe - highly significant - reduction of the relative EC tropism, proving these charge clusters to be essential for EC tropism. CCTA mutations of aa DGE60-62 or RAK163-165 caused a weak - though significant - reduction of the relative EC tropism. Most interestingly with regard to the hypothesis, mutation of the basic motif KKHKR154-158 caused an intermediate phenotype with a 10fold - highly significant - reduction of the relative EC tropism. In order to exclude the possibility that the reduced EC tropism of mutants was due to second site mutations, revertant viruses were generated. Again, markerless mutagenesis was employed to restore the wild type sequence of the respective charge cluster. All revertants were indistinguishable from the wild type virus HCMV-TB40-BAC4 with regard to relative EC tropism (Fig. 3C), thus proving that the phenotypic changes were specifically caused by the mutations of the respective charge clusters.

Evaluation of the focus expansion capacity in EC monolayers independently confirmed the phenotypes of the various mutants (Fig. 4). While CCTA mutations of aa EIR72-74, HSLTR82-86 or EADGR104-108 had completely destroyed the capacity of focal spread in EC monolayers, the virus with the KKHKR154-158 mutation showed an intermediate
phenotype of 16 cells per focus as compared to 72.5 cells per focus with wild type virus. All other mutations had weak or no effects on the focus expansion capacity in EC monolayers when compared to the wild type HCMV-TB40-BAC4, a finding which was also consistent with the results regarding the relative EC tropism of cell free virus preparations (summarized by color codes in figure 1). As the intermediate phenotype of mutant HCMV-BAC4-UL128ccta154-158 was most promising with regard to the aim of a fine tuning of the EC tropism, this mutant was chosen for further modifications.

**Targeted adjustment of endothelial cell tropism by varying mutations in charge cluster pUL128\textsubscript{147-165}**

The KKHKR\textsubscript{154-158} sequence in the center of charge cluster pUL128\textsubscript{147-165} was particularly interesting not only because the respective CCTA mutant showed an intermediate phenotype but also because of the remarkable accumulation of five basic amino acids in a row. The fact that replacement of five basic amino acids caused a 10-fold reduction of EC tropism raised the possibility of further fine tuning of the EC tropism by varying the number of mutations at this site. To test this hypothesis an additional mutant BAC was generated where only the central 3 amino acids (KHK\textsubscript{155-157}) of this pentapeptide sequence were replaced with alanines by markerless mutagenesis of the wild type BAC. Virus was then reconstituted by transfection of the mutant BAC in HFFs and virus stocks were harvested for analysis of EC tropism. The resulting mutant HCMV-BAC4-UL128ccta155-157 (KHK\textsubscript{155-157}) was compared to HCMV-BAC4-UL128ccta154-158 (KKHKR\textsubscript{154-158}) and to wild type virus by analyzing both the relative EC tropism of cell-free virus preparations and the focus expansion capacity in the coculture assay. In support of our hypothesis, the KHK\textsubscript{155-157} mutant showed a significantly reduced relative
EC tropism in the cell-free infection mode as compared to wild type virus, but this reduction was weaker than with the KKHKR_{154-158} mutant and the difference between both mutants was highly significant (Fig. 5A). The coculture-based assay confirmed these results, with regard to the mean values of the various viruses: the FE_{HUVEC} of the KHK_{155-157} mutant was reduced by 20% when compared to wild type virus whereas the KKHKR_{154-158} mutant showed a 3-4-fold reduction in the focus expansion assay. Due to the higher variation of this assay, though, the difference between the KHK_{155-157} mutant and the wild type virus was not significant (Fig. 5B). In synopsis of both assays, however, it is obvious that the degree of reduction of EC tropism could be adjusted by varying the number of amino acids exchanged in the UL128_{154-158} pentapeptide.

**Discussion**

Being part of a pentameric complex together with the basic fusion machinery gH/gL (15, 22) the UL131A-128 gene products are essential for HCMV infection of epithelial cells and ECs (1, 7, 11, 32) and contribute to cell-associated spread in fibroblasts (14). Recently, the virus-inhibitory and cell-surface-binding properties of soluble pUL128 have suggested a direct and functional interaction of pUL128 and ECs (18). By mutational scanning of UL128 in the viral context we have now identified several charge clusters that are relevant with regard to cell tropism. While three adjacent charge clusters in the central part of the protein sequence were absolutely essential for infection of ECs, both the N-terminal part and the C-terminal part contained charge clusters that contributed partially to infection of ECs. Based on the aforementioned reports on interactions of pUL128 (18, 22) it is tempting to speculate that the N-terminal part and the C-terminal part of the protein each bind to viral complex partners while the central core region
interacts with cellular surface structures. Ryckman et al. have demonstrated direct interactions of UL128 both with gL and UL130, each of which bound independently to gH (22). This implies that loss of one anchoring would still allow pUL128 to stay in the complex, which corresponds well to the phenotype of the C-terminal or N-terminal mutants. Mutation of aa DGE\textsubscript{60-62} to AGA\textsubscript{60-62} (representing aa 33 to 35 after cleavage of the predicted signal peptide) only weakly reduced infection of EC. Likewise, mutation of aa KKHKR\textsubscript{154-158} to AAAAA\textsubscript{154-158} and aa RAK\textsubscript{163-165} to AAA\textsubscript{163-165} caused an intermediate or weak reduction of EC tropism. We hypothesize that these charge clusters are involved in binding to UL130 and gH, whereas the central core region of pUL128 interacts with target cells. Patrone et al. have reported that pUL128 binds to the cell surface and upon binding induces conformational changes in the viral envelope protein gB, finally resulting in fusion of the viral envelope with cellular membranes (18). This corresponds well to the null phenotype of our viruses carrying CCTA-mutations of aa EIR\textsubscript{72-74}, HSLTR\textsubscript{82-86} or EADGR\textsubscript{104-108}. In particular, the dual function of cell binding and conformational change may be reflected by involvement of multiple charge clusters.

While being speculative at the moment, these considerations allow functional predictions regarding binding to pUL130, gL and ECs that are now analyzed in an ongoing study with the respective mutants.

Aim of our study was to perform a functional scanning of UL128 in the viral context and to use the results as a basis for targeted modulation of the endotheliotropic phenotype. This was not trivial as conventional cloning techniques insert marker sequences at the mutation site which would disrupt the respective open reading frames. Even with more advanced techniques leaving only FRT sites or loxP sites after removal of the selection
marker, a contribution of the specific mutations could not be dissected from effects of the "mutagenesis scars". Only the recent introduction of markerless mutagenesis techniques for BAC-cloned herpesvirus genomes (29, 33) enabled such a functional mapping of a protein of interest in the viral context. Still, a comprehensive screening of all possible mutants would be infeasible and yield irrelevant results when mutations just destroy the backbone of the protein rather than its specific interaction sites. To increase the probability of targeting exposed sites of pUL128 we relied on charge-cluster-to alanine scanning, an approach which has been extensively applied for mutational analyses of vaccinia viruses (e.g. (6, 12)) but also for isolated herpesvirus proteins (3) in the past. The combination of both approaches allowed the systematic mapping of functionally relevant sites in an HCMV protein within the viral context, which to our knowledge has not been performed with HCMV before. Importantly, a number of charge cluster mutations in UL128 had no effect on the endotheliotropic phenotype, which can serve as internal controls corroborating the specificity of the approach. In addition, the perfect restoration of EC tropism in each of the revertants excluded that any of the phenotypic changes were due to second site mutations, which proves the reliability of the method. After this successful "proof of principle", any desirable viral protein can now be analyzed similarly in the viral context.

The combination of markerless mutagenesis with CCTA scanning appears particularly suitable for the generation of mutants with highly specific phenotypes. Mere deletions often result in a null phenotype, which precludes a more detailed analysis of the interaction of viral proteins with their interaction partners, especially when a protein interacts with multiple partners, like UL128 does. In this context, CCTA mutations of aa
DGE\textsubscript{60-62} or RAK\textsubscript{163-165} are particularly interesting for further functional analyses. It is tempting to presume a specific interaction of these charge clusters with gL and pUL130, respectively, which would suggest an additive effect of a dual mutation. Regarding our assumption that CCTA mutagenesis in the viral context would enable the targeted fine tuning of EC tropism, mutation of aa KKHKR\textsubscript{154-158} was most interesting. The respective virus mutant displayed an intermediate phenotype, i.e. a 10fold reduced relative EC tropism, which was the ideal starting point for a further modification. As predicted, decreasing the number of mutated amino acids within this charge cluster modified the phenotype, resulting in a 3-4fold reduced EC tropism of the KHK\textsubscript{155-157} mutant. This finding opens the possibility for the intended generation of HCMV strains with desired phenotypic properties, which may e.g. be applied for the rational design of an attenuated HCMV live vaccine.

In summary this study demonstrates a novel approach to identify functionally relevant sites within a protein in the context of replicating HCMV. Specifically, multiple charge clusters within pUL128 have been identified which contribute to EC tropism to a variable degree and may be assigned to the various interaction partners of this protein in future studies. More generally, the combination of markerless mutagenesis with CCTA scanning can facilitate the generation of HCMV strains with a desired degree of attenuation through targeted modulation of potential HCMV virulence factors.

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References


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Legends to figures:

Fig. 1: Charge clusters in the amino acid sequence of pUL128 of TB40-BAC4. Charged amino acids are indicated by bold letters. Charge clusters are highlighted in grey. Motifs that were chosen for charge-cluster-to-alanine mutagenesis (i.e. amino acids 18-21, 38-42, 60-62, 72-74, 82-86, 104-108, 136-139, 154-158, 163-165) are marked by boxes. Colors indicate the phenotype of the respective CCTA mutants: green = no phenotype, yellow = weak phenotype, orange = intermediate phenotype, red = severe phenotype. The cleavage site of the predicted signal peptide is marked by a backslash (\).

Fig. 2: Restriction pattern analysis of UL128ccta mutant BACs. The integrity of the TB40-BAC4-UL128ccta mutant BACs was controlled by digestion of 3µg of BAC DNA with 20 U EcoRI restriction enzyme and fragment separation on a 0.8% agarose gel. A: Restriction pattern after 24h. B: Restriction pattern after 36h. M: Marker, 1: TB40-BAC4, 2: TB40-BAC4-UL128ccta18-21, 3: TB40-BAC4-UL128ccta38-42, 4: TB40-BAC4-UL128ccta60-62, 5: TB40-BAC4-UL128ccta72-74, 6: TB40-BAC4-UL128ccta82-86, 7: TB40-BAC4-UL128ccta104-108, 8: TB40-BAC4-UL128ccta136-139, 9: TB40-BAC4-UL128ccta154-158, 10: TB40-BAC4-UL128ccta155-157, 11: TB40-BAC4-UL128ccta163-165.
Fig. 3: Relative endothelial cell tropism of UL128ccta mutants. Fibroblasts (HFFs) and endothelial cells (HUVECs) were infected by various UL128ccta mutants at an infection multiplicity of 0.7 infectious units/cells. One day after infection viral immediate-early antigens were detected by indirect immunofluorescence staining (Cy3, red nuclear signals). All cell nuclei were counterstained with DAPI (blue nuclear signals). A: By way of example one mutant without phenotype (UL128ccta18-21), one mutant with intermediate EC tropism (UL128ccta154-158) and one mutant with a severe reduction in EC tropism (UL128ccta82-86) are shown in comparison to the wild type TB40-BAC4. B: The relative EC tropism of all UL128ccta-mutants was determined as the ratio of infection efficiency in HUVECs / infection efficiency in HFFs. Bars represent mean values of at least 3 experiments (standard error of the mean is indicated with each bar). Asterisks indicate whether the difference between a mutant and the wild type virus is significant (*) or highly significant (**). C: Relative EC tropism of UL128ccta-revertants as described for panel B.

Fig. 4: Focus expansion capacity of UL128ccta-mutants in endothelial cell monolayers. Productively infected fibroblasts were cocultured with an excess of uninfected endothelial cells (indicator cells) for 5 days, fixed and stained for viral immediate-early antigen (Cy3, red nuclear signals). Nuclei were counterstained with DAPI. By way of example one mutant without phenotype (UL128ccta18-21), one mutant with intermediate EC tropism (UL128ccta154-158) and one mutant with a severe reduction in EC tropism (UL128ccta82-86) are shown in comparison to wild type TB40-BAC4.
Fig. 5: Fine tuning of the EC tropism by varying the number of mutated amino acids in cluster 154-158. The endothelial cell tropism of wild type TB40-BAC4, UL128ccta155-157 and UL128ccta154-158 was compared by two independent assays. Asterisks indicate whether differences are significant (*) or highly significant (**). **A:** Relative endothelial cell tropism of cell free virus preparations as determined by the ratio of infection efficiencies in endothelial cells and fibroblasts. For each bar the standard error of the mean is indicated. **B:** Focus expansion capacity in endothelial cell monolayers as determined by evaluation of the focus size after 5 days of coculture. For each bar the standard error of the mean is indicated.
Table 1: Overview of TB40-BAC4-UL128ccta mutants and their respective amino acid exchanges

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<td>re CAGCATATAGCCCATTTTAGCGCGACCGACACATCCAGCAGACCAGT</td>
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<tr>
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<td>TB40-BAC4-UL128ctta155-157</td>
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<td>TB40-BAC4-UL128ctta163-165</td>
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<tr>
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<td>re ATGGGCTATAATGCTGAGTGGGCTGAGGCTGAGGAGT</td>
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Note: fo = forward primer; re = reverse primer; upper case letters = HCMV homology; lower case letters = homology to template plasmid pEP-Kan-S; bold letters = sequence of interest